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Footprinting at low temperatures: evidence that ethidium and other simple intercalators can discriminate between different nucleotide sequences

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ABSTRACT

Footprinting experiments employing DNAase I have been performed at 4°C. At this temperature several simple intercalating ligands, including both ethidium and proflavine, can be seen to induce marked changes in the pattern of cleavage. From an analysis of the changes in patterns of digestion by DNAase I we deduce that ethidium binds best to regions of mixed nucleotide sequence, especially those containing alternating purines and pyrmidines. Binding seems to be weakest to polydA sequences which consequently appear as regions of relatively enhanced cleavage. Attempts to reproduce these changes using DNAase II as a footprinting tool were unsuccessful.

INTRODUCTION

A wide variety of techniques have been used to study the intercalative binding of ethidium to DNA. They have yielded much information on the structure and dynamics of the complex formed between the ligand and various nucleic acids [1-7]. However, the origin and extent of any sequence selectivity in the interaction of ethidium with DNA remain uncertain, although its binding affinity for a variety of DNAs is known to be relatively independent of their gross base-composition [1,2]. It has been demonstrated that the drug displays a clear preference for binding to Py(3'-5')Pu sequences compared to their respective Pu(3'-5')Py isomers [4]. This has been confirmed by competitive binding studies with natural DNAs showing that at low levels of binding ethidium does not compete with actinomycin D, which binds specifically to the dinucleotide step GpC, whereas competitive binding does occur with the synthetic actinomycin analogue actinomine, which binds equally well to GpC and CpG [5]. Studies with synthetic polynucleotides have revealed a considerable variation in their capacity to bind ethidium. In general, the drug binds well to DNAs containing alternating purines and pyrimidines but much less well to homopolyermic purine-pyrimidine duplexes [6,8,9]. It is especially noteworthy that it binds very poorly to polydA.polydT in marked contrast to the heteroduplex polyrA.polydT to which it binds very well [6], suggesting that

differences in binding affinity arise not merely from any simple sequence selectivity but from a preferential recognition of different nucleic acid conformations [6,9]. A preference for binding to one region of DNA over another can also be inferred from the observation that at low levels of occupancy ethidium binds to certain DNAs in a cooperative fashion [7]. NMR studies on short DNA fragments of mixed sequence have also suggested that ethidium displays a slight preference for binding adjacent to G.C base pairs [10].

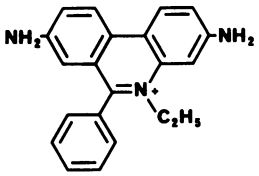
In this paper we report the results of footprinting studies on the selective binding of ethidium to various DNAs of defined sequences. Footprinting is a method combining the enzymatic cleavage of DNA in the presence and absence of a selective drug with analysis of the products on sequencing gels [11-13]. Sites to which the ligand is specifically bound are visualised at single-bond resolution as gaps in the autoradiographs of denaturing polyacrylamide gels. This technique has been widely employed for investigating the binding of highly-specific antibiotic ligands to DNA [11-19], whereas experiments with simpler compounds have not previously yielded clear footprinting patterns [12,20]. The difference could reflect a genuinely lower order of selectivity in the binding of the smaller ligands or it could be a result of their faster dissociation from the DNA, enabling them to escape detection by the usual footprinting probe. Indeed this apparent lack of selectivity is an important property of MPE-Fe (II), a reactive derivative of ethidium which is one of the most useful footprinting tools [12,14,17]. While this reagent is generally assumed to be relatively non-specific it is evidently not totally sequence neutral since it does not produce a completely even ladder of cleavage products [14].

In this paper we describe a simple extension of the standard footprinting technique rendering it useful for investigating the selective binding of small ligands, such as ethidium, to DNA. By working at low temperatures (4°C) the dissociation of the ligand from DNA is slowed sufficiently to permit a meaningful DNAase I footprinting analysis.

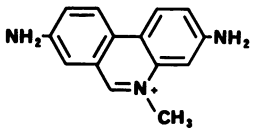
MATERIALS AND METHODS

Drugs and enzymes

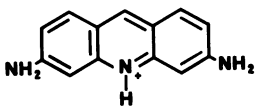
Ethidium bromide was purchased from Sigma Chemical Co. Proflavine hemisulphate was obtained from BDH. Des-phenyl dimidium (MB 2421) was a gift from Drs R. Slack and S.S. Berg, May and Baker Ltd., Dagenham, England. The structures of these DNA-binding compounds are illustrated in Figure 1. Stock



Ethidium



Des-phenyl dimidium



Proflavine

Figure 1. Structures of ethidium, des-phenyl dimidium and proflavine.

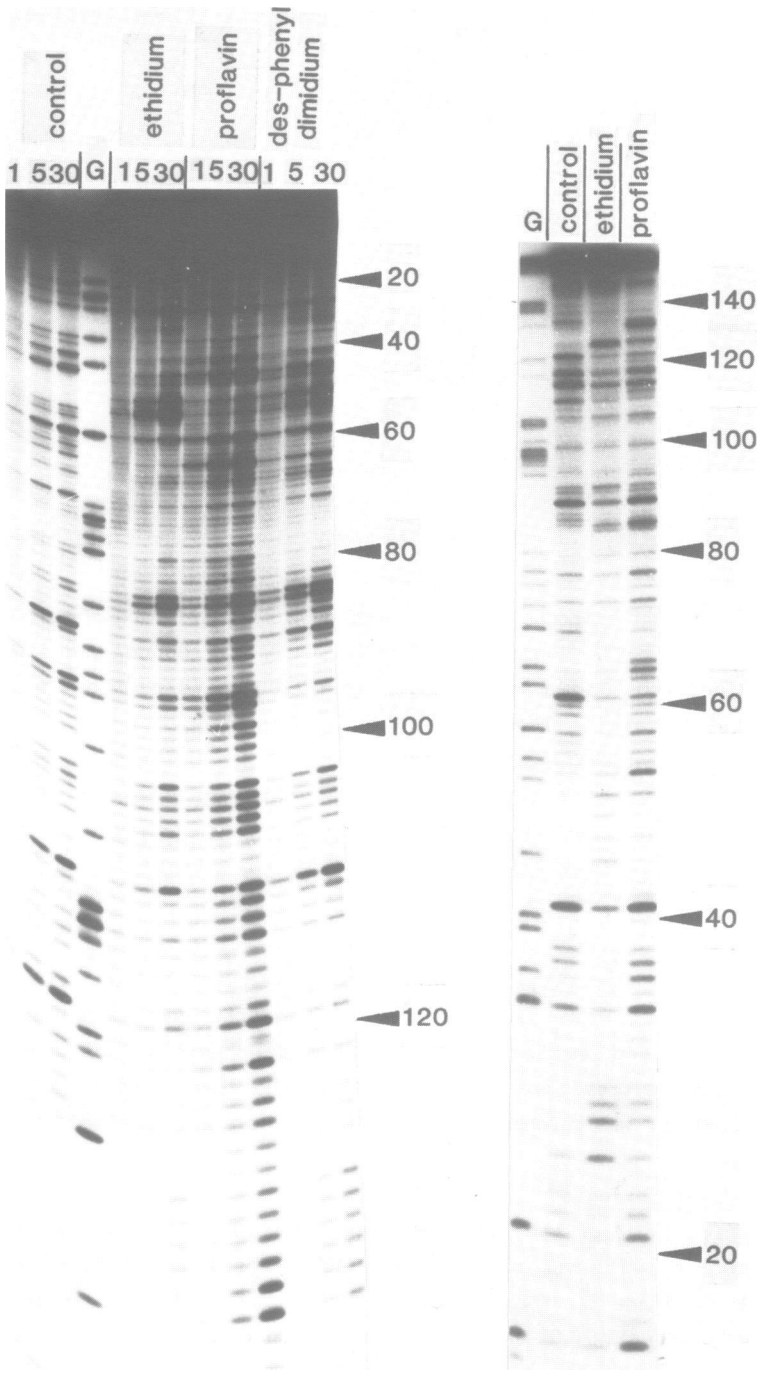
solutions of each compound were prepared by direct weighing and dissolved in 10mM tris-HCl, pH 7.5 containing 10mM NaCl. Deoxyribonuclease I (DNAase I) was obtained from Sigma and prepared as a 7200 units/ml stock solution in 0.15M NaCl containing 1mM $MgCl_2$. It was stored at -20°C and diluted to working concentrations immediately before use. The digestion buffer used for dilution contained 20mM NaCl, 2mM $MgCl_2$ and 2mM $MnCl_2$. Deoxyribonuclease II (DNAase II) was obtained from PL Biochemicals and prepared as a 2000 units/ml stock solution in 10mM ammonium acetate, pH 5.6, containing 0.2mM EDTA and stored at -20°C.

DNA fragments

The 160 base pair tyrT DNA, 166 base pair pTyr2 DNA and 119 base pair Xbs1 DNA fragments were isolated from plasmids pKM -98, pMLB1048 and pXbs1 respectively and selectively labelled at their 3' ends with reverse transcriptase as previously described [18]. The 49 base pair pUC13 multiple cloning fragment was purchased from PL Biochemicals and labelled as previously described [18].

DNAase I footprinting

Samples (3µl) of labelled DNA (approximately 10 pmoles in base pairs) were incubated with 5µl of the drug (5-40µM) for 30 minutes, then digested with 2µl of DNAase I. For experiments at 4°C the final concentration of the enzyme was typically 0.01 units/ml for the control untreated DNA, 0.2 units/ml for complexes containing 20µM proflavin and 0.7 units/ml for DNA in the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ethidium
proflavin
140
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100
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presence of 20μM ethidium. Experiments at 37°C were performed as previously described using five times more enzyme in the drug-treated samples than in the control. Samples (3μl) were removed from each reaction mixture after 1, 5 and 30 minutes digestion and the reaction stopped by adding 2.5μl of 80% formamide containing 0.1% bromophenol blue and 10mM EDTA. These were heated at 100°C for at least 2 minutes prior to electrophoresis.

DNAase II footprinting

Samples (3μl) of labelled DNA were incubated with 6μl of drug dissolved in 10mM ammonium acetate pH 5.6 before digestion with 3μl DNAase II (final concentration 500 units/ml) at 4°C. Samples (3μl) were removed after 1, 5, 30 minutes and the reaction was stopped by adding 2.5μl of 80% formamide and freezing on dry ice. Samples were heated at 100° for 3 minutes prior to electrophoresis.

Gel electrophoresis

Products of enzyme digestion were fractionated on polyacrylamide gels (0.3mm thick) prepared in Tris-borate-EDTA buffer containing 7M urea, 12% w/v for pUC13 DNA and 10% for tyrT, Xbs1 and pTyr2 DNA fragments. Gels were fixed in 10% acetic acid, transferred to Whatman 3MM paper, dried under vacuum and subjected to autoradiography at -70°C with an intensifying screen.

Densitometry

Autoradiographs were scanned using a Joyce-Loebl microdensitometer to produce profiles from which the relative intensity of each bond was measured. The chemical identity of digestion products was assigned by reference to the known sequences and dimethylsulphate-piperidine tracks specific for guanine. DNAase I products bear a 5' phosphate group and migrate in phase with guanine markers during electrophoresis while DNAase II products carry a 5' hydroxyl group and migrate up to 1.5 bonds more slowly than the markers. This difference in mobility becomes larger as the chains grow shorter [21,22].

The data are expressed in terms of fractional cleavage (f) = A_i/A_t as previously described [15,16,18,19], where A_i is the area under band i and A_t is the sum of the intensities under all bands in any gel lane. They are

Figure 2. DNAase I footprinting of ethidium on the 160 base pair tyrT DNA fragment at 4°C. (a) Left hand panel: Autoradiograph of the DNAase I digest of the 3'-end labelled top strand in the presence of ethidium, proflavine and des-phenyldimidium each at 20μM. Time in minutes after the addition of enzyme is shown at the top of each gel lane. (b) Right hand panel: DNAase I digest of the 3'-end labelled bottom strand in the presence of ethidium and proflavine, each at 20μM, at 4°C. Each reaction was stopped after 5 minutes with the enzyme concentration adjusted as described in the text. Tracks labelled 'G' are dimethyl sulphate-piperidine markers specific for guanine.

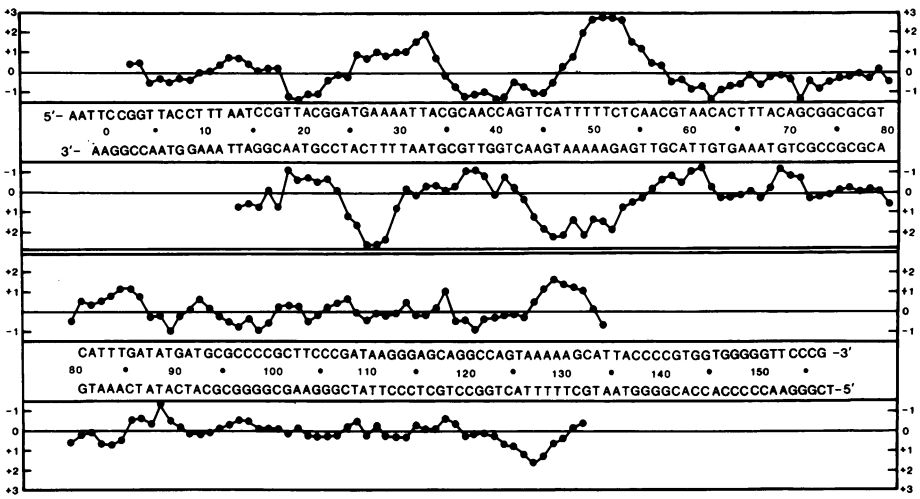


Figure 3. Differential cleavage plot showing differences in the relative probability of cutting of tyrT DNA by DNAase I in the presence of 20μM ethidium at 4°C. Positive values indicate relative enhancement negative values blockage.

presented in the form of $\ln(f_{antibiotic}) - \ln(f_{control})$, representing the differential cleavage at each bond relative to that in the control. Positive values indicate bonds which are relatively enhanced, negative values show those which are blocked. It is worth emphasizing that for the ligands investigated in this paper the cutting at all bonds is inhibited (so that higher enzyme concentrations are required in drug treated samples to see any cutting at all). The differential cleavage plot then represents the changes in the relative probability of cutting at each bond, indicating which positions are inhibited to a greater or lesser degree than the average.

RESULTS

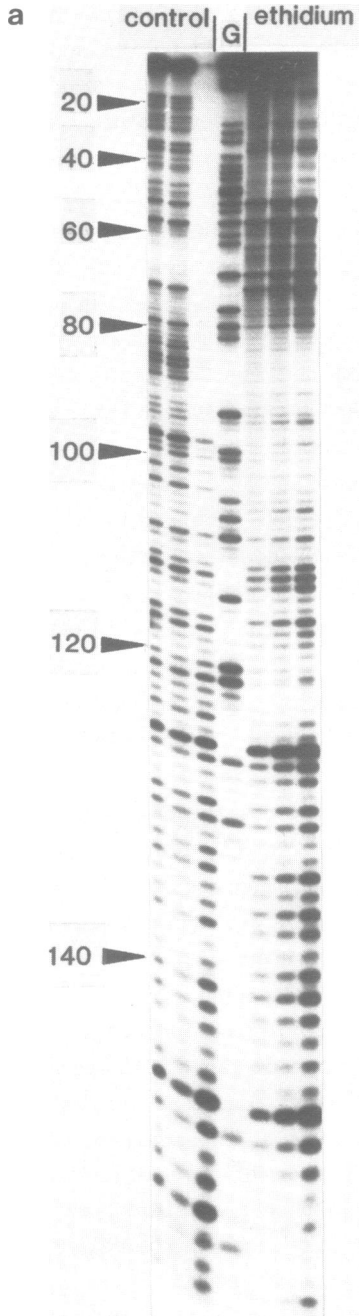
Preliminary footprinting experiments with 20μM ethidium and tyrT DNA revealed a generally inhibited pattern of DNAase I cutting with only a few small changes in relative band intensities. At this ligand concentration increasing the enzyme concentration four to five fold restored the extent of digestion in the drug treated samples to that seen in the control. Higher drug concentrations required a greater enzyme concentration, or longer digestion, to produce a similar extent of cleavage, although the drug-induced cutting pattern remained constant. We found, however, that repeating the

experiment at 4°C (and adjusting the enzyme concentration accordingly) produced changes in the cleavage pattern which were much more pronounced, though still occurring in the same regions. This improvement in the quality of the results by working at lower temperature could be explained in several ways, the most likely being an increase in the persistence time of the drug-DNA complex.

The results of DNAase I footprinting experiments performed at 4°C with various ligands in the presence of tyrT DNA are presented in Figure 2. Looking first at the results for ethidium we can see that the relative pattern of cleavage differs substantially from that in the control. For the labelled upper strand (left panel) the most obvious changes are the enhancements visible around positions 30 and 50, neither of which regions are cut particulary well in the control. For the labelled lower strand (right panel) similar relative enhancements can be seen around positions 28, 48 and 130. Both labelled strands show regions of sharply reduced cleavage in the presence of ethidium. These differences are more clearly illustrated by the differential cleavage plot, presented in Figure 3. In this plot positive values indicate those bonds at which cutting is enhanced while negative values indicate relative blockages. The differential cleavage pattern is staggered across the two DNA strands by about three bonds in the 3' direction, as previously observed for DNAase I footprinting with other antibiotics [15,16,18,19], presumably because DNAase I cuts by binding to phosphate groups that lie in close proximity across the double-helical minor groove. Regions on both strands which are relatively protected can be seen around positions 20, 40, 60, 70 and 90 with enhancements around positions 29, 50 and 130.

Also displayed in Figure 2 are the results of similar experiments with proflavine and des-phenyl dimidium [23]. The des-phenyl analogue produces a pattern of enhancement and protection virtually identical to that seen with ethidium. The effect of proflavine appears intermediate, lying part way between the ethidium and control lanes. It seems then that the changes are not peculiar to ethidium, which possesses a bulky phenyl side group, but can be produced by other even simpler intercalators. The changes are, however, not a necessary consequence of intercalation since m-AMSA (4'-[9-acridinylamino] methanesulphon-m-anisidide) and its ortho derivative o-AMSA [24] produced no detectable changes in the relative cutting pattern at all, even at concentrations as high as 100μM.

Inspection of Figure 3 reveals that the regions of relatively enhanced cleavage (those where ethidium protects less well) always occur in runs of A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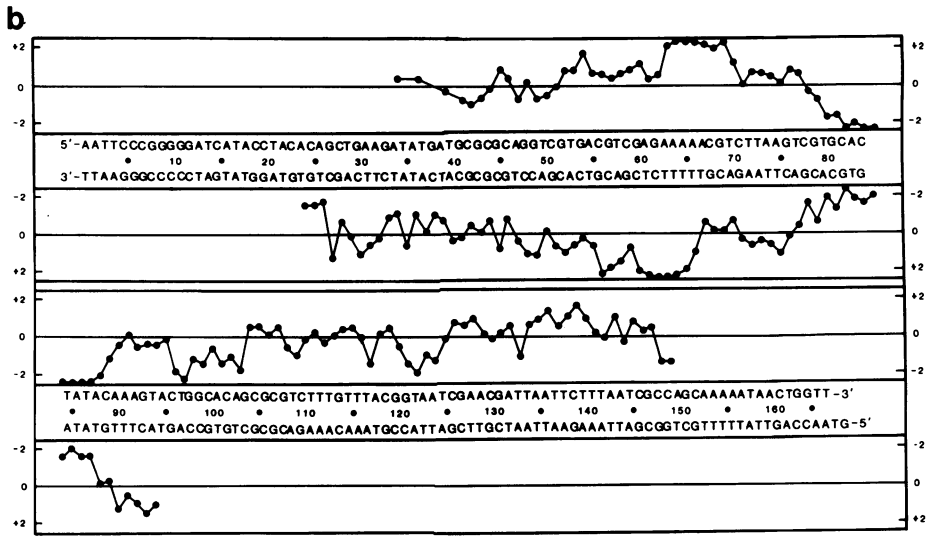
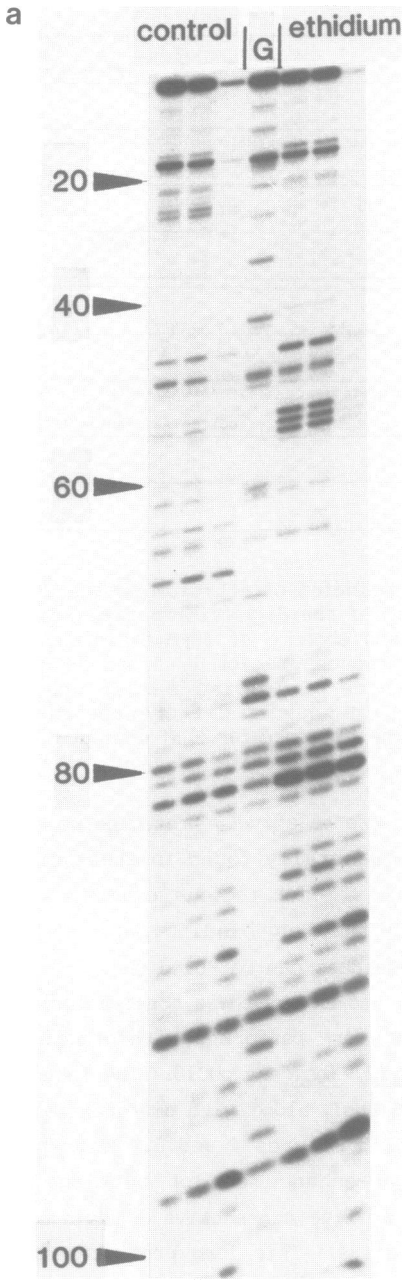


Figure 4. DNAase I footprinting of ethidium on the 166 base pair pTyr2 DNA at 4°C. (a) Autoradiograph of the digestion pattern of the 3'-end labelled top strand in the presence and absence of 20μM ethidium. Each set of three lanes represents digestion by the enzyme for 1, 5 and 30 mins. The track labelled 'G' is a dimethyl sulphate-piperidine marker specific for guanine. (b) Differential cleavage plot showing differences in relative probability of cutting by DNAase I in the presence of 20μM ethidium.

or T. The regions which are relatively protected do not share any such simple characteristic but are generally found in areas of mixed sequence. It is worth noting that the two GC-rich regions around positions 75 and 100 are not selectively affected by the drug binding, so that a simple preference for binding adjacent to G residues can be ruled out.

In order to clarify and extend these observations similar low temperature footprinting experiments were performed with ethidium using three other DNA fragments designated pTyr2, Xbsl and pUC13. The footprinting patterns for one strand each of pTyr2 and Xbsl, along with differential cleavage plots for both strands, are presented in Figures 4 and 5. With pTyr2 DNA (Figures 4a,b) there is an impressive enhancement of cutting around positions 60-65 corresponding to a run of five consecutive A residues and a clear protection between positions 78 and 104. This long protected site contains two regions of nearly alternating purines and pyrimidines, GTGCACTATACA between positions 78 and 89 and GTACTGGCACA between positions 92 and 102. With Xbsl DNA (Figures 5a,b) two clear regions of enhanced cutting are visible around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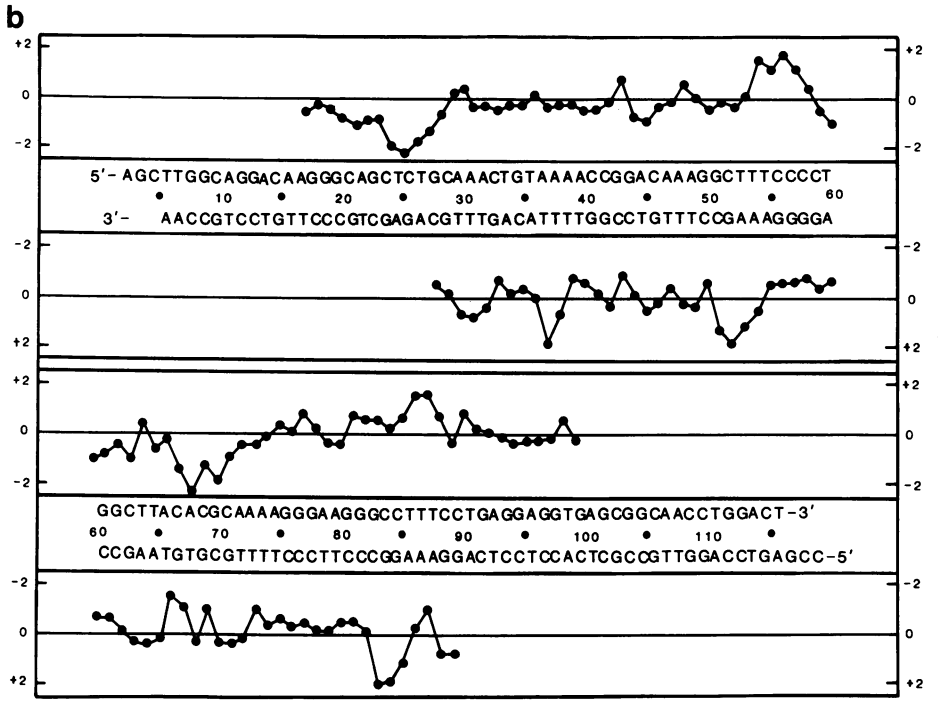

Figure 5. DNAase I footprinting of ethidium on the 119 base pair XbsI DNA fragment at 4°C. (a) autoradiograph of the 3' end labelled top strand in the presence and absence of 20 μM ethidium. Each set of three lanes represents digestion by the enzyme for 1, 5 and 30 mins. The track labelled "G" is a dimethylsulphate piperidine marker specific for guanine. (b) Differential cleavage plot showing differences in relative probability of cutting by DNAase I in the presence of 20μM ethidium.

positions 52 and 87, both of which correspond to short runs of A and T. Strong protection is afforded around positions 67 and 25. The former again occurs in a region of alternating purines and pyrimidines TACACGCA while the latter contains a run of alternating C and T residues. Experiments with the short pUC13 DNA fragment, which contains only one short run of alternating purines and pyrimidines between positions 10-14 (TGCA) [18] revealed a weak block at this position but a much stronger relative protection between positions 20 and 29 in the sequence ACTCTAGAGG corresponding to short runs of alternating C and T residues (or G and A).

Since these experiments were all performed with DNAase I as a

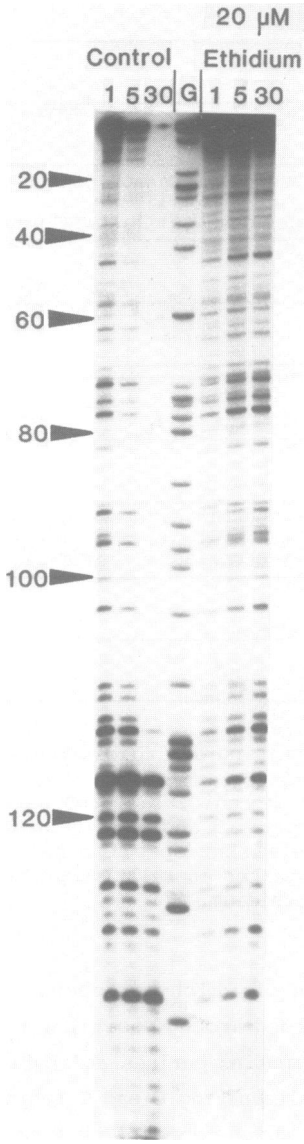

Figure 6. Autoradiograph of the DNAase II digestion pattern of tyrT DNA (top strand) in the presence of 20 μM ethidium at 4°C. Time in minutes after the addition of enzyme is shown at the top of each gel lane. The track labelled "G" is a dimethylsulphate-piperidine marker specific for guanine. The DNAase II reaction products bear a 5' hydroxyl group and migrate up to 1.5 bonds more slowly than the marker, which carries a 5' phosphate group. The numbering scheme corresponds to the identity of the bonds shown in Figures 2 and 3(b), as for the DNAase I cleavage reaction.

footprinting agent we decided to confirm the changes using DNAase II, which has occasionally been used as a footprinting tool [16] and has very different sequence and structural requirements [21]. The results of such an experiment with 20μM ethidium and tyrT DNA labelled on the upper strand are presented in Figure 6. The only major change produced by the drug is the blockage between positions 120 and 125 which corresponds to a region hardly affected in the DNAase I footprinting experiments (Figure 3). Interestingly, none of the regions which ethidium protects from DNAase I are noticeably affected in the DNAase II digestion pattern. These differences must reflect the very different structural features of DNA recognised by the two enzymes. DNAase I appears to bind across the DNA minor groove, cutting best in those regions where the width of the groove is about average (10-12A); whereas it seems that DNAase II cutting, which is anticorrelated across the two DNA stands, occurs best whenever the same-strand phosphate-phosphate spacing is small, typically in runs of G or G and A [21]. It is possible then that any observed alterations in susceptibility to DNAase II attack induced by ethidium binding do not result from simple steric blockage but arise from a longer-range distortion of the DNA structure to a form less readily recognised by the enzyme. It should be noted that the blockage between positions 120 and 125 corresponds to the only region on the "top" strand which is cut well by DNAase II [22].

DISCUSSION

Footprinting at low temperatures

The results presented in this paper demonstrate that it is possible to generate specific DNAase I footprinting patterns with quite simple ligands such as ethidium merely by performing experiments at 4°C. Why should this be? Firstly we should note that the DNAase I cleavage pattern of DNA alone changes as a function of temperature, so that the "gaps" where the enzyme attacks poorly appear more pronounced at lower temperatures [22]. If these changes in the native cutting pattern reflect certain alterations in the DNA structure then it is possible that working at lower temperatures might exaggerate the ability of ligands to discriminate between various sequences. This possibility is rendered even more likely if we postulate that the selectivity of ethidium results from some capacity to discriminate between different types of DNA structures rather than between different sequences themselves. Put another way it is conceivable that the sequence-selectivity of ethidium is temperature dependent. While such factors must affect the observed pattern of

protection we feel that a more important reason for the clearer picture at low temperatures arises from the slower dissociation of the ligand. At moderate temperatures the ligand dissociates quite rapidly from DNA so that any blockages are short-lived and therefore not readily detected by the enzyme. On cooling the reaction down the persistence time of the drug-DNA complex increases and the location of the ligand can more easily be seen as blocks in the DNAase I cleavage pattern.

It is worth emphasising that according to this argument the selectivity of drugs as determined by footprinting will tend to reflect binding to those sites with the slowest dissociation rates, and not necessarily those with the tightest binding constants. Although these two parameters are dependent on each other, it is known that closely related compounds having similar binding constants can display markedly different kinetic profiles as is the case for actinomine and actinomycin D [25]. However, similar experiments with other highly selective ligands (such as echinomycin and actinomycin) have revealed little or no change in the footprinting pattern at lower temperature, despite the observation that their binding to natural DNA is characterized by multiple types of binding sites with a variety of dissociation rate constants [26,27]. In contrast to the experiments described in this paper, low temperature studies with actinomycin D and echinomycin were successfully performed using the same enzyme concentration in antibiotic-treated as well as control samples to provide similar extents of digestion.

Selectivity of ethidium

Whatever the ultimate explanation may be, it is clear that at 4°C ethidium displays some real ability to discriminate between different DNA sequences. What may be the basis for this sequence preference? The clearest changes are observed in those regions to which ethidium does not bind very well and which thus appear as relative enhancements of cutting. These changes always occur at runs of A or T in all the DNAs examined. This finding is entirely consistent with experiments using synthetic polynucleotides which have consistently shown very low binding constants for ethidium and polydA.polydT [6,8]. It is interesting that our experiments confirm that, whatever the factors which impede binding of ethidium to polydA.polydT, the same or similar behaviour persists when short runs of A, 3 or more nucleotides long, are found in natural DNA fragments.

Before considering the probable nature of sequences to which ethidium binds best it is worth noting that the cleavage pattern must in part reflect the reactivity of the cutting reagent towards its substrate. Consequently,

the relative binding affinities of sequence-selective ligands for various nucleotide sequences represent only one of the parameters that can affect the pattern, as discussed above. Thus it is important that any observed changes be carefully compared with appropriate control reactions. This objective is effectively achieved by constructing the differential cleavage plots from a detailed densitometric analysis of each band in both control and drug treated lanes.

It can be seen that the sequences to which ethidium binds best are generally rich in alternating purines and pyrimidines, especially those of mixed base composition. Neither GCGC nor ATAT constitute good binding sites (cf. positions 75 and 87 respectively in tyrT DNA). Arguably the clearest region of selective protection is seen with pTyr2 DNA, consisting of a long gap between positions 80 and 105 containing two regions of alternating purines and pyrimidines. However, other regions are sometimes well protected as for instance around position 25 of Xbs1 DNA in the sequence CTCT.

Since there appears to be no simple rule governing the type(s) of sequence(s) to which ethidium binds best it seems sensible to propose that the ligand can discriminate between different local DNA structures, rather than recognising any specific arrangement of bases. If this is the case then we may expect a similar pattern for most simple intercalators, reflecting how easy it is (or not) to insert an inert wedge into each local DNA structure. We have found this to be true for both proflavine and des-phenyldimidium, the intermediate pattern seen with the former probably reflecting its faster dissociation rate. Many, though not all, of the blockages produced by the larger intercalator nogalamycin also occur in positions similar to those seen with ethidium [18]. This again suggests that these ligands seek out certain structural or dynamic peculiarities of the local DNA helix.

Conformational changes

We have previously reported that when sequence-selective ligands bind to DNA they can induce structural changes in neighbouring regions which render them more sensitive to cleavage by DNAase I [15,16,18]. Can this occur when ethidium binds to DNA? It is impossible to answer this question from the present data because, unlike the situation in previous experiments, we were forced to employ a much higher enzyme concentration (100-fold; i.e. 4.6 $\log_e$ units) for the drug treated samples than the drug-free control in order to produce similar extents of digestion. The strong, apparently enhanced, cutting which is seen in runs of A and T in the presence of ethidium could result either from structural changes caused by drug binding to adjacent

regions, or merely because the affected regions exclude the ligand so that increasing the enzyme concentration compensates for their much lower susceptibility in the control. The situation is further complicated in that any structural changes induced in these runs of A or T could render them susceptible to drug binding, a factor which might explain the apparent cooperativity observed in the binding of ethidium to certain natural DNAs [7].

Footprinting techniques

The preferred binding sites for ethidium, as assessed by DNAase I footprinting, apparently escape detection by DNAase II. Although this second enzyme has only rarely been used as a footprinting tool [16] the present observations serve to emphasize that the protection pattern depends, to some degree, on the nature of the cleaving agent. It has previously been suggested tht DNAase I and MPE-Fe (II) provide different information about drug-DNA interactions; the former is more useful for detecting weakly bound sites, while the latter is more accurate for determining the actual site size and location for small molecules interacting with DNA [14]. Thus the type of information obtained from a footprinting analysis depends on the nature of the probe employed. In the present instance such changes in the DNAase II cutting pattern as may be induced by ethidium cannot be interpreted through lack of detailed information about the precise structural requirements for DNA cleavage by this enzyme.

The selective effects of ethidium observed in these experiments have obvious implications for the interpretation of footprinting experiments performed using MPE-Fe (II) as the cleavage reagent [12,14,17]. Because this synthetic cleavage agent has a central ethidium-like portion it would be expected to interact with different sequences in a fashion similar to the parent compound. As previously noted this ligand is not entirely sequence-neutral [14] and a close inspection of patterns determined at 37°C reveals markedly reduced cutting in runs of A or T [17], which can be enhanced by binding of echinomycin to adjacent regions [17]. This serves to emphasise the importance of comparison with control, drug-free lanes even when using a simple chemical cleavage agent which is supposed to generate a more or less even ladder of bands, just as is the case in experiments where enzymes are employed. Indeed it would be interesting to investigate MPE-Fe (II) cleavage at 4°C when we would anticipate that the pattern of cleavage should appear as the inverse of that seen in this paper.

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